INFECTION AND IMMUNITY, Mar. 1980, p. 837-841
0019-9567/80/03-0837/05$02.00/0

In Vitro Studies of Interaction of Rickettsia and Macrophages: Effect of Ultraviolet Light on *Coxiella burnetii* Inactivation and Macrophage Enzymes

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The inactivation of *Coxiella burnetii* in suspension or in cultures of guinea pig peritoneal macrophages by ultraviolet (UV) light was studied. The effect of UV treatment on the activity of macrophage organelle marker enzymes and their subsequent equilibration in linear sucrose gradients was also determined. It was shown that UV treatment of 600 $\mu W/cm^2$ for 15 s at a distance of 10 cm inactivated *C. burnetti*, either in suspension (10^8 organisms per ml) or within guinea pig peritoneal macrophages. Similar UV treatment had little effect on the activity or equilibration of macrophage organelle marker enzymes in linear sucrose gradients. However, longer exposure caused considerable inactivation of these enzymes.

While designing experiments to determine the intracellular fate of *Coxiella burnetii* in guinea pig peritoneal macrophages, it was necessary as a safety precaution to inactivate ingested rickettsiae without significantly inactivating macrophage enzymes. These enzymes would subsequently be used as markers for determining the distribution of cellular organelles on linear sucrose gradients after homogenization and fractionation of macrophages containing ingested rickettsiae. Therefore, inactivation of the rickettsiae without inhibition of macrophage organelle marker enzymes was a prerequisite. Chemical fixation, alcohol denaturation, heat, and a number of other conventional methods of inactivation were known to affect adversely enzyme activity. Previous studies have shown that rickettsiae in suspension could be inactivated by exposure to ultraviolet (UV) light (1, 14). However, the effect of UV light on rickettsiae growing in macrophages and on the activity of macrophage organelle marker enzymes was unknown. This paper describes a method for the inactivation of *C. burnetii* in suspension or in guinea pig peritoneal macrophages without considerably altering the activity of several marker enzymes for macrophage subcellular components.

MATERIALS AND METHODS

Chemicals. [*methyl*-^{3}H]thymidine (47 Ci/mmol) and [*G*-^{3}H]adenosine 5'-monophosphate (13.5 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, Mass.). Scintisol Complete was obtained from Isolab, Inc. (Akron, Ohio). Ultrapure, ribonuclease-free sucrose was obtained from Schwarz/Mann (Orangeburg, N.Y.). The 4-methylumbelliferyl substrates used in the biochemical assays were purchased from Koch-Light Laboratories, Ltd. (Colnbrook, Buckinghamshire, England). All other chemicals were of analytical grade when available and were obtained from commercial sources.

Animals. Outbred male Hartley strain guinea pigs, weighing approximately 350 to 450 g, and outbred white mice [Tac:(SW)fBr], weighing 18 to 22 g, were obtained from Buckberg Lab Animals (Tompkins Cove, N.Y.).

Culture and labeling of rickettsial stock suspension. The third egg passage of the Henzerling strain of *C. burnetii* in phase I and the 88th egg passage of the phase II Nine-Mile strain were grown in chicken embryo cells as previously described (10). Nine days after inoculation the medium was removed; 50 ml of fresh medium containing 10 μCi of [*methyl*-^{3}H]thymidine was added to each roller bottle. Rickettsiae were harvested 24 h later as previously described (10). Samples were pooled, and rickettsial counts were determined by the method of Silberman and Fiset (16).

Preparation of guinea pig peritoneal macrophages. Peritoneal exudate cells were collected 4 days after guinea pigs were injected intraperitoneally with 20 ml of a 1.5% sodium caseinate solution. Cells were harvested and processed according to the method of David et al. (6). Approximately 5×10^6 macrophages were dispensed into petri dishes (15 cm in diameter; Falcon Plastics, Oxnard, Calif.) and incubated at 37°C for 2 h in a humid atmosphere of air containing 5% CO_2. Nonadherent cells were removed by two washes with Hanks balanced salt solution (HBSS), and then 5 ml of fresh Earle 199 medium was added.

Infection of macrophages. Rickettsial suspensions containing approximately 10^8 organisms of either phase I or phase II *C. burnetii* per ml were incubated for 30 min at 37°C in Earle 199 medium containing 10% heat-inactivated anti-phase I or anti-phase II *C. burnetii* guinea pig serum (1:1,000 microagglutination titer). Serum-treated rickettsiae were then added to

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macrophage cultures in a ratio of 100 rickettsiae per macrophage and incubated at 37°C for 60 min. The inoculum was removed, and macrophage cultures were washed three times with 20 ml of HBSS.

UV light irradiation. Control and infected macrophage cultures, washed twice with 10 ml of HBSS, were overlaid with 10 ml of HBSS so that the depth of the overlay was approximately 1 mm. The cultures were then placed on ice and exposed to UV light (600 $\mu W/cm^2$, Sylvania G15T8 germicidal tube) at a distance of 10 cm for up to 10 min. After exposure to UV light, macrophages were scraped off the petri dish with a rubber-tipped scraper. The suspension medium was collected; an additional 10 ml of HBSS was added to each culture dish, and the plates were scraped again. The macrophages contained in the pooled suspension medium were then either assayed directly for marker enzymes or homogenized and fractionated as described below.

Three milliliters of rickettsial suspension containing 10^8 organisms of phase I or II *C. burnetii* per ml was dispensed into each petri dish (8 cm in diameter), which resulted in suspensions approximately 1 mm in depth. The suspensions were then exposed to UV light for 15 s at a distance of 10 cm.

Test for photoreactivation. Suspensions of phase I or II *C. burnetii* (10^8 organisms per ml), UV treated as described, were left exposed to light under laboratory conditions for 3 days and then titrated in white mice as described below.

Titration of rickettsiae. One-milliliter amounts of various dilutions of rickettsial suspensions and infected macrophages were inoculated intraperitoneally into four white mice for each dilution point. The animals were bled 21 days later; sera were checked for specific antibodies by indirect immunofluorescence (4).

Homogenization of guinea pig peritoneal macrophages. Irradiated or control macrophage suspensions were sedimented at 480 × *g* for 5 min in a Sorvall RC3-B refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The cellular pellet was homogenized in 0.25 M sucrose containing 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid and 3 mM imidazole hydrochloride buffer (pH 7.5) with a syringe by seven passages through a 12-inch-long, 25-gauge cannula (5). The nuclei and unbroken cells were sedimented by differential centrifugation at 480 × *g* for 5 min. The supernatant (designated as cytoplasmic extract) was saved, and the pellet was rehomogenized and recentrifuged as described above. This process was repeated four times; the cytoplasmic extracts from the centrifugations were combined.

Fractionation of cytoplasmic extracts. A portion of the pooled cytoplasmic extract was layered on a sucrose gradient containing 3 mM imidazole hydrochloride buffer (pH 7.5) and extending linearly with respect to volume from a density of 1.10 to 1.25. The gradient, contained in a Beckman Quickseal tube, rested on a 5-ml cushion of 1.3 M buffered sucrose. Centrifugation was in a Beckman V50 rotor for 90 min at 40,000 rpm in a Beckman L2-65 B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Following centrifugation, 12 fractions of approximately equal volume were pumped from the centrifuge tube into tared tubes. The fractions, maintained at 4°C, were weighed, and their contents mixed. The density of each fraction was determined on a Bausch & Lomb Abbe III refractometer. Each fraction was then assayed for enzymatic activity as described previously (5).

Determination of radioactivity. For determination of radioactivity, 0.2 ml of each fraction was mixed with 0.8 ml of water. To this, 20 ml of Scintisol Complete was added. Samples were counted in a mark III liquid scintillation spectrophotometer (Searle & Co., Des Plaines, Ill.). Counting efficiency was approximately 41%, and differential quench from vial to vial was not encountered.

Presentation of results. The distribution of phase I and II *C. burnetii* in linear sucrose gradients and the distribution patterns of enzymes after fractionation of cytoplasmic extracts in linear sucrose gradients are presented in the form of histograms constructed as previously described (2, 3, 7, 11).

RESULTS

UV light irradiation. Approximately 10^8 viable organisms of phase I or II *C. burnetii* in suspension were exposed to UV light for 15 s. After treatment, 1.0-ml amounts of various dilutions (10^0 to 10^{-8}) were titrated intraperitoneally in white mice and failed to elicit detectable serum antibodies at 21 days. In addition, when similar suspensions were treated with UV light, exposed to light under laboratory conditions for 3 days, and then titrated as described, no serum antibodies were detected. Thus, photoreactivation did not occur. Similar results were obtained when macrophages were infected with 10^8 viable organisms of phase I or II *C. burnetii* (UV treated and titrated in the same manner) per ml. Previous studies have shown that one phase I organism or one phase II (EP-88) organism can infect and seroconvert white mice (unpublished data). These results suggest that exposure to UV light for 15 s inactivates phase I and II *C. burnetii* both in suspension and in macrophage cultures.

Effect of UV treatment on the activity of macrophage organelle marker enzymes. The activity of macrophage organelle marker enzymes was considerably decreased when macrophage cultures were exposed to UV light for up to 10 min (Fig. 1). The range of inhibition at 10 min of UV exposure was from 51% for malate dehydrogenase to 100% for alkaline α-glucosidase and *N*-acetyl-β-glucosaminidase (Fig. 1). However, after 15 s of exposure, inhibition did not exceed 35% (5′-nucleotidase, Fig. 1); in fact, the activities of both lysosomal enzyme markers (α-D-galactosidase and *N*-acetyl-β-glucosaminidase) increased by 50 and 20%, respectively (Fig. 1). The reasons for activation of lysosomal enzymes by UV light of short duration are not

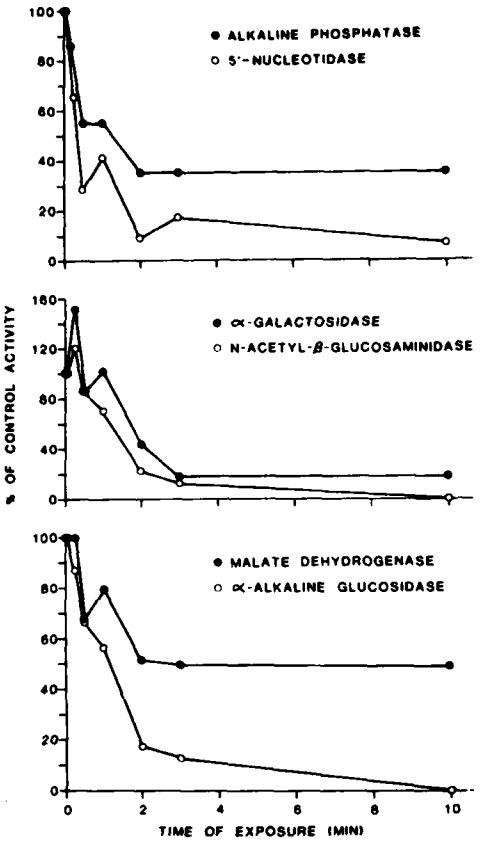

FIG. 1. *Effect of UV treatment on activity of macrophage enzymes. UV treatment and enzyme assays were as described in the text. Results are those of one representative experiment.*

apparent. It does not appear to be due to the unmasking of structure-linked latency exhibited by lysosomes (8), since both enzyme assays contained detergent concentrations sufficient to unmask latent activity.

Fractionation of cytoplasmic extracts. Exposure of macrophages to UV light for 15 s did not appear to alter the buoyant properties of macrophage organelles. The distribution and mean equilibrium density of marker enzymes after centrifugal equilibrium on sucrose gradients remained essentially unchanged after UV exposure (Fig. 2).

Equilibration of *C. burnetii* on linear sucrose gradients and determination of enzyme activity. Radiolabeled phase I and II *C. burnetii* organisms, irradiated for 15 s, were layered on sucrose gradients and centrifuged to equilibrium. Phase I had an equilibrium density in the alkaline sucrose of 1.24 (Fig. 3), a value which is in agreement with previous reports (17, 18). Phase II was found to have an equilibrium density of 1.21. When gradient fractions were assayed for malate dehydrogenase, 5'-nucleotidase, alkaline α-glucosidase, α-D-galactosidase, alkaline phosphatase, and *N*-acetyl-β-glucosaminidase, no detectable quantities of these macrophage marker enzyme activities were found in either the phase I or the phase II *C. burnetii* gradients.

DISCUSSION

The present study shows that phase I and II *C. burnetii* organisms either in suspension or growing within cultured guinea pig peritoneal macrophages are inactivated by brief exposure to UV light. Treatment of *C. burnetii* with UV decreases the risk to individuals working with this highly virulent species. Since certain procedures required for subsequent studies on the interaction of *C. burnetii* with macrophages could cause aerosolization, working with inactivated organisms was desirable. It must be noted, however, that photoreactivation of UV-inactivated organisms can take place (15). We have shown, however, that exposure of phase I or II *C. burnetii* to light under laboratory conditions for 3 days did not result in photoreactivation.

We have shown that exposure of macrophage cultures to UV light for 15 s does not appreciably inactivate marker enzymes for macrophage organelles or their subsequent equilibration on linear sucrose gradients. These results suggest that UV treatment does not adversely affect the conformation of these proteins or alter the permeability to sucrose of lysosomes, microsomes, or mitochondria. Since these enzymes retain most of their activity after UV treatment, they may be used confidently as markers for the localization of cellular constituents on linear sucrose gradients after fractionation of macrophage homogenates. It is now possible to determine the intracellular distribution and fate of *C. burnetii* in guinea pig peritoneal macrophages by using analytical methods of subcellular fractionation.

Finally, it has been shown that rickettsiae contain a number of different enzymes (12, 13). Our results suggest that neither phase I nor phase II *C. burnetii* contains detectable amounts of the enzymes assayed in these experiments. However, it must be noted that these preparations were UV treated before assay. The effect of this UV treatment on the rickettsial enzymes is not known. The absence in phase I and II *C. burnetii* of detectable quantities of enzymes chosen as markers for macrophage organelles greatly simplifies and facilitates fractionation studies. It eliminates the requirement for cor-

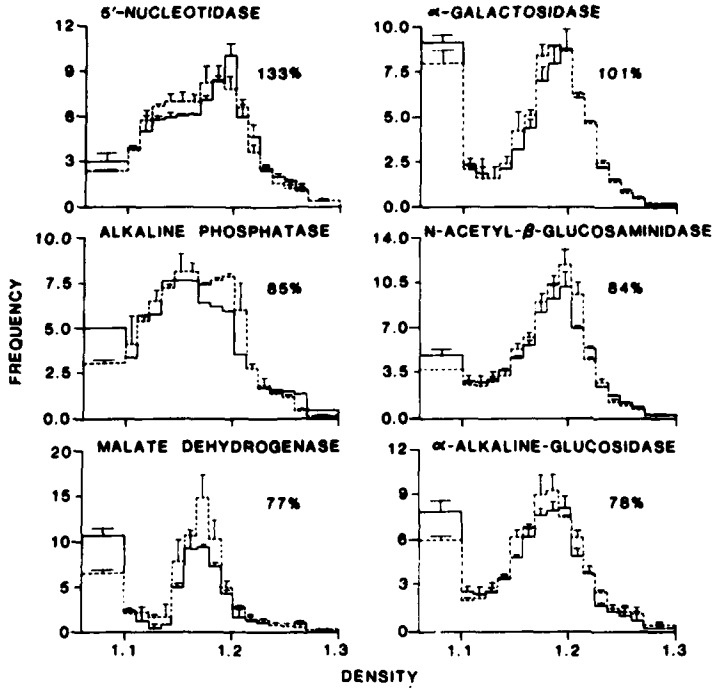

FIG. 2. *Distribution profiles of constituents from cytoplasmic extract of control (——) or UV-treated (---) guinea pig peritoneal macrophages. Macrophages were treated with UV light for 15 s as described in the text. Results are presented in the form of normalized and averaged frequency histograms. The density scale, divided into 15 normalized fractions of identical density increments, extends from 1.10 to 1.25. The frequency represents $\Delta Q/(\epsilon Q \Delta\rho)$, where ΔQ is the amount of constituent present within the section and ϵQ is the sum of the amounts found in all the subfractions. The surface area of each histogram bar then gives the fractional amount of constituent present within each normalized fraction. $\Delta\rho$ is equal to 0.0113 density units. Distribution profiles are flanked on either side by blocks arbitrarily constructed over the density spans 1.06 to 1.10 and 1.25 to 1.30 and refer to material recovered above and below the linear limits of the gradient. The total area of each histogram is then equal to 1. Diagrams show averages of results with standard errors of the mean. The percentage in the upper right corner of each histogram represents the recovery of enzyme activity.*

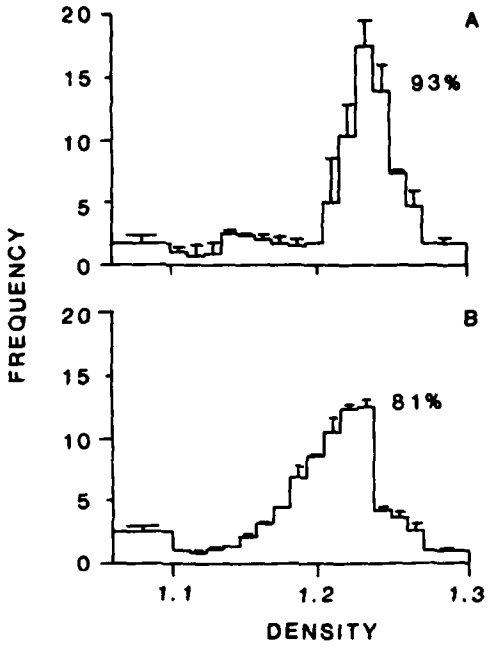

FIG. 3. *Equilibration of radiolabeled phase I (A) and II (B) C. burnetii on sucrose gradients. Results are expressed as described in the legend to Fig. 2. The percentage in the upper right corner of each histogram represents the recovery of the label.*

recting macrophage enzyme activities for those due to rickettsiae.

ACKNOWLEDGMENTS

We thank Paul Merrill, Wayne Rill, Dennis Smith, and Robert Stockman for technical assistance. We gratefully acknowledge the editorial reviews of P. W. Summers, B. Larson, R. Wachter, K. Hedlund, and J. Johnson.

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